



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : C12Q 1/25, G01N 33/53 A61K 35/30	A1	(11) International Publication Number: WO 93/04194 (43) International Publication Date: 4 March 1993 (04.03.93)
<p>(21) International Application Number: PCT/US92/06700</p> <p>(22) International Filing Date: 10 August 1992 (10.08.92)</p> <p>(30) Priority data: 744,767 13 August 1991 (13.08.91) US</p> <p>(71) Applicants: REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; Morrill Hall, 100 Church Street, S.E., Minneapolis, MN 55455 (US). PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 124 Mt. Auburn Street, Cambridge, MA 02138 (US).</p> <p>(72) Inventors: MAGGIO, John, Edward ; 480 Washington Street, Brookline, MA 02146-4516 (US). MANTYH, Patrick, William ; 6413 Interlachen Boulevard, Edina, MN 55436 (US).</p>		<p>(74) Agent: HAMRE, Curtis, B.; Merchant, Gould, Smith, Edell, Welter &amp; Schmidt, 3100 Norwest Center, 90 South Seventh Street, Minneapolis, MN 55402 (US).</p> <p>(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).</p> <p>Published With international search report.</p> <p style="text-align: center;">205</p>
<p>(54) Title: LABELLED <math>\beta</math>-AMYLOID PEPTIDE AND ALZHEIMER'S DISEASE DETECTION</p> <p>(57) Abstract:</p> <p>The present invention provides: a labelled <math>\beta</math>-amyloid peptide or active fragment; a composition including the labelled <math>\beta</math>-amyloid peptide or active fragment thereof and a pharmaceutical carrier; a method for labelling the <math>\beta</math>-amyloid peptide or an active fragment thereof, and methods of using the labelled peptide or peptide fragment for detecting or monitoring Alzheimer's disease in a patient.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TC	Togo
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain			US	United States of America

1

LABELLED  $\beta$ -AMYLOID PEPTIDE AND ALZHEIMER'S  
DISEASE DETECTION

5                    STATEMENT REGARDING FEDERALLY-FUNDED  
                    AND SPONSORED RESEARCH

                    This invention was made with government support  
from the National Institutes of Health, NIH grant NS-  
23970, NS-22961, and NS-26312. The government may have  
10 certain rights in this invention.

BACKGROUND OF THE INVENTION

                    Alzheimer's disease is a widespread progressive  
dementia affecting a significant fraction of the elderly  
15 population. While there have been significant advances  
in the research over about the last five years, the  
primary pathology of the disorder remains unknown. The  
behavioral symptoms of Alzheimer's disease are well  
known, and include loss of memory and cognitive  
20 function. The salient pathological symptom of  
Alzheimer's disease at autopsy is the presence in  
certain brain areas of extracellular proteinaceous  
deposits or plaques called amyloid on the basis of their  
staining with various reagents.

25                    The extracellular amyloid is deposited both at  
neuronal and vascular sites, and the density of these  
deposits in the cerebral cortex and blood vessels  
correlates positively with the degree of dementia (D.J.  
Selkoe, Neuron 6: 487 (1991); D.J. Selkoe, Science 248:  
30 1058 (1990); B. Muller-Hill et al., Ann. Rev. Biochem.  
58: 287 (1989); R. Katzman et al., FASEB J. 5: 278  
(1991)). The principal component of both neuritic and  
vascular plaques in Alzheimer's disease is beta-amyloid  
peptide ( $\beta$ -amyloid or A4 peptide), a hydrophobic peptide  
35 of 39-43 amino acids which is encoded by a gene for a  
much larger protein termed the amyloid precursor protein  
(APP). Mature amyloid plaques have a halo of  
degenerating neurons around a core of the  $\beta$ -amyloid  
peptide (R.J. Perry, Br. Med. Bull. 42: 34-41 (1986)).

To date, neither the processing of APP to  $\beta$ -amyloid peptide nor the genesis of the amyloid deposits has been well understood. The characteristics of  $\beta$ -amyloid peptide deposition and the factors that affect it remain  
5 key questions in the pathology of Alzheimer's disease.

At the present time, there is no established test other than brain biopsy for diagnosing Alzheimer's disease antemortem. Further, there is no system to quantify neuropathological changes associated with  
10 Alzheimer's disease. In addition, there is no method that has been developed to screen and evaluate agents that may have unique anti-Alzheimer's disease action. There is also no method for in vitro evaluation of anti-Alzheimer's disease agents that does not require a  
15 sample of patient tissue.

In view of the present lack of knowledge about the development and progression of Alzheimer's disease, there is a need for agents and methods suitable for the diagnosis and detection of Alzheimer's disease. More  
20 particularly, there is a need for compounds and assay techniques that can be employed to screen for potential agents that inhibit or enhance the development of amyloid plaques. Such compounds and methods would be useful in assessing senile plaque formation associated  
25 with the onset and progression of Alzheimer's disease.

#### SUMMARY OF THE INVENTION

These and other objects are achieved by the present invention which is directed to a composition and  
30 method that is useful for studying, detecting and monitoring the progression of Alzheimer's disease in a patient. More specifically, the composition and method of the present invention are useful for detecting and quantitating amyloid deposition in vivo and in vitro.  
35 Further, the present invention provides methods for screening and testing agents which inhibit or enhance amyloid deposition in human tissue.

The present invention provides a labelled  $\beta$ -amyloid peptide useful for detecting Alzheimer's disease and studying Alzheimer's disease-related conditions. A method for obtaining the labelled  $\beta$ -amyloid peptide is also provided. The method employs essentially dry  $\beta$ -amyloid peptide and rapid formation of a labelled  $\beta$ -amyloid peptide. Preferably, the peptide has the amino acid sequence:

H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-OH [SEQ. ID NO:1] or an active fragment of such amyloid peptide. In the preferred embodiment, the  $\beta$ -amyloid peptide is bound to a radioactive label such as radioactive iodine. However, other appropriate labelling agents and techniques, for example, enzymatic or fluorescent labelling of the  $\beta$ -amyloid peptide or active peptide fragment, can be used, either alone or in combination. The labelled peptide can be combined with a pharmaceutically acceptable carrier for in vivo diagnostic and possible therapeutic use.

The present invention relates to various uses of the labelled  $\beta$ -amyloid peptide. One such use is in vitro detection and monitoring of Alzheimer's disease in a patient. This is accomplished by combining a sample of patient tissue with an amount of labelled  $\beta$ -amyloid peptide or active fragment thereof for a period of time effective to allow binding of the labelled peptide or peptide fragment to the tissue. The bound labelled peptide/tissue complex is then detected and, if desired, quantified. In vitro detection and monitoring can be accomplished by numerous techniques, including autoradiographic or homogenate binding assays. Homogenate binding assays can be used to screen for potential therapeutic agents, in particular, the ability of these agents to affect deposition of  $\beta$ -amyloid peptide onto tissue and existing plaques. This includes agents that inhibit or enhance deposition or are capable of breaking up existing plaques. Further, progression

of Alzheimer's disease may be monitored by assaying a later-acquired sample of tissue from a patient earlier tested in the same manner as the earlier-acquired sample. The amount of bound peptide or peptide fragment  
5 in the two tissue samples is compared to provide an assessment as to the development of the disease in a patient.

According to the present invention, in vivo detection of Alzheimer's disease in a patient is also  
10 possible by administering the labelled  $\beta$ -amyloid peptide or active fragment to the patient and detecting the presence of the labelled peptide or peptide fragment bound to the tissue in the patient by known imaging techniques such as positron emission tomography (PET)  
15 imaging.

Another aspect of the invention is an in vitro method for screening agents capable of affecting the aggregation of  $\beta$ -amyloid peptide. The method can be used to evaluate agents that inhibit or enhance  
20 aggregation. This includes an agent's ability to break up and, in certain cases, to inhibit formation or growth of plaques. Agents screened may be of potential use as therapeutic compositions for treatment of Alzheimer's disease. Screening of agents effecting  $\beta$ -amyloid  
25 peptide aggregation can be conducted in a test tube without plaque material. Thus, the present invention provides a technique for assessing agents that affect  $\beta$ -amyloid peptide aggregation that requires no patient tissue sample. In vitro screening of potentially useful  
30 Alzheimer's disease agents is accomplished by combining  $\beta$ -amyloid peptide or an active peptide fragment thereof with the potential aggregation affecting agent to be screened in a solution. The amount of  $\beta$ -amyloid peptide aggregation is then detected and assessed to determine  
35 the effect of the agent on  $\beta$ -amyloid peptide or peptide fragment aggregation. This can be accomplished either in solution, or by filtration, centrifugation and the

like. The aggregation affecting agent to be screened may be combined with the  $\beta$ -amyloid peptide or fragment thereof, either before, at or after the start of the peptide aggregation reaction. Alternatively, the  $\beta$ -amyloid peptide aggregation utilizes an aggregation enhancing agent (e.g., detergent, divalent metal cation) prior to inclusion of the aggregation affecting agent to be screened.

The labelled  $\beta$ -amyloid peptide of the present invention and methods of use described herein provide qualitative and quantitative diagnostic tools for studying and potentially treating Alzheimer's disease. Use of  $\beta$ -amyloid peptide aggregation as a screening tool for compositions having potential therapeutic use provides a previously unavailable technique to study and evaluate potential therapeutic agents without patient tissue.

Other features and advantages of the invention will be apparent from the following detailed description and appended claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graphic representation of competitive inhibition of specific binding of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> by amyloid and tachykinin peptides in homogenates of Alzheimer's disease temporal cortex.

LEGEND:  $\square$   $\beta$ -amyloid peptide<sup>1-40</sup> ( $\beta$ -AP<sup>1-40</sup>);  $\circ$  Dutch  $\beta$ -amyloid peptide<sup>1-40</sup>;  $\square$   $\beta$ -amyloid peptide<sup>25-35</sup>-NH<sub>2</sub>; and  $\circ$  Substance P, Neurokinin A, Neurokinin B,  $\beta$ -amyloid peptide<sup>25-35</sup>-OH, Rat  $\beta$ -amyloid peptide<sup>1-40</sup>.

Figure 2 is an autoradiographic localization of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> binding sites in Alzheimer's disease brain.

Figure 3 is an autoradiographic localization of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> binding sites in the cerebral vasculature of Alzheimer's disease brain tissue.

Figure 4 is graphic depiction of in vitro aggregation of • human  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup>, and • human  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> in the presence of sodium dodecylsulfate (SDS).

5

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides: a labelled  $\beta$ -amyloid peptide or active (i.e., biologically, or chemically active, or shown as positive in an assay) fragment; a composition including the labelled  $\beta$ -amyloid peptide or active fragment thereof and a pharmaceutical carrier; a method for labelling the  $\beta$ -amyloid peptide or an active fragment thereof, and methods of using the labelled peptide or peptide fragment for detecting or monitoring Alzheimer's disease in a patient.

As used herein, the term "aggregation" refers to the tendency of a large molecule or colloidal body to associate together into a mass or body of units or parts.

Labelled  $\beta$ -amyloid peptide or active fragments are used in the methods according to the invention.  $\beta$ -amyloid peptide has a sequence of about 40 amino acids. The exact length of the naturally-occurring peptide may vary from about 39 to 43 amino acids, depending on the presence of ragged ends. The sequence of the 42-mer peptide is H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGGVIA-OH [SEQ. ID NO:2], and the sequence of the 40-mer peptide is H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGGVV-OH [SEQ. ID NO:1]. The 40-mer peptide is preferred in the present invention. However, active fragments having as few as about 5 amino acids and ranging from about 5 to about 43 amino acid units are useful if appropriate labelling and measuring techniques are used to detect a smaller fragment of the 39- to 43-mer peptide. In particular, a peptide fragment derived from the 1-43 amino acid region of  $\beta$ -amyloid peptide and having at least 10 amino acid units, as for example, a fragment



containing the amino acids at about position 25-35, may be used according to the invention.

As used herein, abbreviations for the amino acids are as listed in Table 1, as shown below. In addition, abbreviations for peptide termini are as follows: "H-" means a free amino group, "-OH" means a free carboxyl group, and "-NH<sub>2</sub>" means a carboxyamide. Sequences are numbered from the amino termini with positions indicated by superscripts.

10

Table I. Amino Acid Codes

<u>Single letter Code</u>	<u>3-letter Code</u>	<u>Amino Acid</u>
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

The  $\beta$ -amyloid peptide or active fragment is combined with an acceptable label as described herein.

5 The label can be radioactive, enzymatic, or fluorescent, or any combination thereof. Preferably, a radioactive label such as radioactive iodine-125 is used.

Among isotopes, any radioactive substance that may be incorporated into the peptide or peptide fragment  
 10 may be used. Preferred isotopes include, but are not limited to, <sup>125</sup>I, and <sup>131</sup>I; the latter has a

shorter half-life and higher energy level. Iodine radioisotopes may be incorporated into the peptide or peptide fragment by oxidative iodination. Also, radioactive iodine may be incorporated by use of Bolton-Hunter reagent to add a 3-iodo-4-hydroxyphenylpropionyl or 3,5-diiodo-4-hydroxypropionyl group to a nucleophile in the peptide.

Other isotopes may also be incorporated by reaction with nucleophile groups on peptides. For example, tritium ( $^3\text{H}$ ) can be incorporated by reaction with propionyl-N-hydroxysuccinimide, or radioactive sulfur ( $^{35}\text{S}$ ) can be incorporated by similar reagents. Radioactive phosphorus ( $^{32}\text{P}$ ) may be incorporated by enzymatic methods. Additionally, various radioactive metal ions, such as  $^{99\text{m}}$ technetium, may be incorporated into  $\beta$ -amyloid peptide or fragments thereof if an appropriate chelating group is added first.

For detection in in vitro assays according to the present invention, enzyme labelling is also useful. Among the preferred enzyme labels are peroxidases such as horseradish peroxidase (HRP), or phosphatases such as alkaline phosphatase.

Modifying the peptide or peptide fragment by adding an antigenic group that will bind with an antibody allows indirect detection of the peptide or peptide fragment itself. For example, the antigen digoxigenin can be linked to an oligonucleotide or peptide, and then visualized with a labelled digoxigenin-specific antibody, or labelled anti-antibody.

Although less sensitive than radioisotopes, fluorophores may also be incorporated into the peptide and detected according to known fluorescent detection techniques. Examples of suitable fluorophores include fluorescein, rhodamine, Texas Red, and the like.

Direct or indirect chemiluminescent labels may also be used according to the invention, such as

dioxetanes. For example, the peptide would be modified with a group that is capable of emitting light as it decomposes.

In addition, an avidin-biotin system may be used to detect the peptide or peptide fragment in an in vitro assay. For example, the peptide or fragment may be functionalized with biotin, and avidin or streptavidin added to detect the protein or fragment.

In vitro methods of detecting Alzheimer's disease according to the present invention combine an amount of sample of tissue obtained from a patient with an amount of labelled  $\beta$ -amyloid peptide or active fragment thereof. The tissue sample may be obtained from any tissue in which the growth of amyloid plaques may occur, including, for example, the nasal epithelium, skin and tissue obtained from portions of the brain such as the cerebral cortex, hippocampus and amygdala, and the like. Preferably the tissue sample used is about 1-20ug/assay tube for tissue prepared in thin section which is preferably about 5-15 $\mu$ m thick, and about 5-50mg/assay tube for tissue prepared as a homogenate.

Submicrogram amounts of the labelled  $\beta$ -amyloid peptide or active fragment thereof, for example, about 0.1 to 10ng of  $^{125}$ I radiolabelled  $\beta$ -amyloid peptide or fragment thereof, is added to each tissue sample for a time effective for the labelled peptide or peptide fragment to bind with the tissue sample. Preferably, the binding reaction time is about 1 to 5 hours, more preferably about 2 hours under the experimental conditions described herein. The time will vary depending on the specific experimental conditions, as will be understood by one skilled in the art. After reaction of the tissue sample with the labelled peptide or fragment, the tissue sample is preferably washed with an appropriate buffer to remove unbound labelled peptide. Homogenized tissue samples are preferably filtered prior to the washing step.

The assay preferably includes a negative control, for example, normal tissue in which binding of  $\beta$ -amyloid peptide is substantially negligible or about 10000 CPM (5% of the total isotope) under the experimental conditions described herein for homogenate binding assays, or less than about 25% of that exhibited in individuals with clinically diagnosed Alzheimer's disease (see Table II, below). The assay may further include a positive control of, for example, tissue that is positive for Alzheimer's disease.

Table II. Summary of clinicopathological features of control and Alzheimer's disease patients compared with the concentration of plaques detected with the  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>I-40</sup> technique.

For tissue with identification numbers (ID#) ending with "-D", the CERAD clinical, neuropsychological and neuropathological was used to confirm the diagnosis of Alzheimer's disease (J.C. Morris et al., Neurology 39: 1159 (1989); S.S. Mirra et al, Neurology 41: 479 (1991), the disclosures of which are incorporated by reference herein. The diagnosis of Alzheimer's disease for tissue with identification numbers (ID#) ending with "-R", was based on clinical assessment by the primary physician and neuropathological examination.

ID#	Age/Sex	PM Interval <sup>1</sup>	Diagnosis <sup>2</sup>	Region <sup>3</sup>	P <sup>4,6</sup>	BV <sup>5,6</sup>
H 001-D	76yrs/F	49 Minutes	AD	T	++++	++
H 002-D	84yrs/F	48 Minutes	AD	F	++++	+
H 003-D	79yrs/M	53 Minutes	AD	T	++++	++++
H 004-D	67yrs/F	25 Minutes	AD	F,T	++++	-
H 005-D	78yrs/F	46 Minutes	AD	T	++++	++
H 006-D	79yrs/M	63 Minutes	AD	T	++++	+
H 017-R	73yrs/F	13 Hrs. 22 Min.	AD	T	++	
H 020-R	63yrs/M	5 Hrs. 22 Min.	AD	T	++++	+
H 023-R	70yrs/F	5 Hrs. 30 Min.	AD	T	++++	
H 007-D	59yrs/F	100 Minutes	Control	F,T	-	-
H 008-D	63yrs/F	103 Minutes	Control	T	-	-
H 009-D	59yrs/F	80 Minutes	Control	T	-	-
H 010-D	70yrs/M	135 Minutes	Control	T	-	-
H 019-R	75yrs/F	10 Hrs. 33 Min.	Control	T	-	-
H 022-R	63yrs/M	5 Hours	Control	T	+	-
H 018-R	75yrs/M	13 Hours	Parkinson's	T	+++	-
H 021-R	60yrs/M	11 Hrs. 20 Min.	Parkinson's	F	-	-

<sup>1</sup> PM = post-mortem

<sup>2</sup> AD = Alzheimer's disease.

<sup>3</sup> T = temporal cortex; F = frontal cortex.

<sup>4</sup> P = parenchyma of cerebral cortex.

<sup>5</sup> BV = blood vessel in cerebral cortex.

<sup>6</sup> concentration of plaques observed in P or BV: (-), not detectable; (+), light; (++) , moderate; (+++) , dense; (++++), very dense.

The in vitro detecting and monitoring techniques according to the present invention can be qualitative or quantitative. The presence of tissue-bound labelled peptide or peptide fragment may be detected according to known techniques appropriate for the particular labelling agent and method used (e.g., radioisotope, fluorophore, enzyme, antigen), the tissue sample type (e.g., homogenate, thin slice), the particular peptide or fragment used (e.g.,  $\beta$ -amyloid peptide<sup>1-40</sup>,  $\beta$ -amyloid peptide<sup>25-35</sup>), and other factors of the assay. In addition, the method of detecting radioactive isotopes will vary according to the isotope and its corresponding energy level. For example, a gamma counter is capable of detecting <sup>125</sup>iodine, but not tritium (<sup>3</sup>H) or <sup>35</sup>sulfur.

Where radiolabelling is used to label the peptide or fragment, the peptide/tissue complex may be detected by

various known radioisotope detection techniques. For example, positron emission tomography may be used to detect isotopes that emit positrons such as radioactive  $^{18}\text{F}$ fluorine or  $^{11}\text{C}$ carbon, gamma counters to detect

5 radioactive  $^{125}\text{I}$ iodine, and scintillation counting methods in the case of tritium ( $^3\text{H}$ ). Nuclear magnetic resonance imaging may also be used, in which case the label would contain a magnetically active particle.

Autoradiography is preferably used to visualize  
10 radiolabelled peptides or peptide fragments in tissue sections, and a radiation counter such as a gamma counter or scintillation counter preferred to detect radioisotopes in tissue samples prepared as a homogenate.

15 In vivo detection and monitoring of Alzheimer's disease includes administering the labelled  $\beta$ -amyloid peptide or active fragment thereof to a patient in an amount effective to bind with tissue evidencing the presence of, or susceptible to, Alzheimer's disease.  
20 Like in vitro detecting methods, the presence of the labelled peptide or peptide fragment bound to tissue in the patient is detected by a known detecting technique that is appropriate to the tissue sample type, the particular peptide or fragment used, the labelling  
25 method used, and other such factors unique to the particular assay being performed.

For medical imaging, the label should be detectable outside of the body. Preferably, the label is a positron emitting radioisotope with a relatively  
30 short half-life, such as  $^{11}\text{C}$ carbon or  $^{18}\text{F}$ fluorine. Such an isotope may be imaged by positron emission tomography, or PET scanning. Magnetic resonance imaging may also be used, in which case the label would include a magnetically active particle.

35 The present invention also provides useful methods to detect, monitor and screen potential therapeutic agents for affecting Alzheimer's disease.

In particular, methods for in vitro screening of agents that are capable of inhibiting or enhancing the aggregation of  $\beta$ -amyloid peptide or active fragments thereof, including the ability to break up and, in certain cases, to inhibit formation or growth of plaques, are provided. According to one method, potential therapeutic agents are placed in competition with labelled  $\beta$ -amyloid peptide in a solution with sample patient tissue, and the effect of the test agent on  $\beta$ -amyloid peptide binding to same tissue is quantified.

Another method is based on the finding that  $\beta$ -amyloid peptide will self-aggregate (i.e., aggregation of  $\beta$ -amyloid molecules based solely on concentration) in solution. This method is particularly advantageous since no patient tissue is required. The aggregation of  $\beta$ -amyloid peptide is dependent primarily on the concentration of the peptide or peptide fragment. For example, an about  $10^{-4}$  molar aqueous buffer solution of  $\beta$ -amyloid peptide<sup>1-40</sup> will commence self-aggregation within a period of about 5 to 30 minutes. At lower concentrations,  $\beta$ -amyloid peptide aggregation may take from about 1 to 5 hours or longer.

In a  $\beta$ -amyloid peptide aggregation screening test,  $\beta$ -amyloid peptide or a peptide fragment thereof is combined with an acceptable buffer or solvent and an agent to be tested. After a specified period, for example 1 to 2 hours, the amount of aggregation is determined. The amount of aggregation can also be periodically monitored over a set time period. More specifically, after a period of time effective to allow aggregation of the peptide or peptide fragment in the solution, for example about 5 to 60, or preferably about 15 to 30 minutes, a potential aggregation affecting agent is added. Alternately, the aggregation affecting agent to be screened may be added at or before the start of aggregation of the peptide. The inhibiting or

enhancing effect of the agent is subsequently determined.

To enhance aggregation of the peptide or fragment, an aggregation enhancing or promoting agent may be combined with the peptide or peptide fragment prior to addition of the aggregation affecting agent. For example, the enhancing agent may be a small amount of pre-formed aggregate of the peptide or peptide fragment, a small amount of purified amyloid plaque derived from Alzheimer's disease tissue, or a substance capable of expediting the aggregation, as for example, about 0.01 to 2% of a detergent such as digitonin, sodium dodecylsulfate (SDS), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) such as CHAPS available from Sigma Chemical Company (St. Louis, Missouri), or octoxynol such as Triton X-100 available from Sigma Chemical Company (St. Louis, Missouri). With respect to deposition of the labelled peptide on tissue plaques, an amount of about 0.1 to 50 millimolar of a metal ion such as manganese ( $Mn^{+2}$ ) or zinc ( $Zn^{+2}$ ) can act as an enhancing agent. The aggregation enhancing agent is added to the peptide/fragment solution in an amount effective to initiate or promote aggregation of the peptide or peptide fragment.

In yet another embodiment, the invention provides a pharmaceutical composition for in vivo use in detecting Alzheimer's disease in a human tissue. The composition contains labelled  $\beta$ -amyloid peptide or active peptide fragment thereof, in a pharmaceutically-acceptable carrier of the type appreciated by those of skill in the art. The composition contains the labelled peptide or peptide fragment in an amount effective to bind to tissue evidencing the presence of, or susceptible to, Alzheimer's disease, when administered in vivo.



The labelled  $\beta$ -amyloid peptide or peptide fragment is useful to detect or quantify the presence of, or tissue susceptibility to, Alzheimer's disease in human tissue. With respect to an in vitro tissue binding assay, the amount of labelled peptide or fragment is effective to bind with tissue evidencing the presence of, or susceptibility to, Alzheimer's disease. Such a binding assay can be used to test agents that may be useful anti-Alzheimer's disease compositions.

10        The aggregation assay described herein provides a technique to screen potential therapeutic agents. In an aggregation assay, the  $\beta$ -amyloid composition will contain the peptide or fragment in an amount effective to self-aggregate, or an amount of  $\beta$ -amyloid peptide together with the chosen aggregation enhancing agent.

15

#### EXAMPLE 1

##### 20        PREPARATION OF LABELLED $\beta$ -AMYLOID PEPTIDE

A radiolabelled amyloid peptide,  $^{125}\text{I}$ -labeled  $\beta$ -amyloid peptide<sup>1-40</sup>, was synthesized for use in determining binding properties of human  $\beta$ -amyloid peptide tissues in homogenates, and to characterize binding to localize tissue sites with which the peptide interacts in thin sections of normal or Alzheimer's disease tissue including central nervous system and vascular tissue.

25

30

Preparation of peptide. Unlabelled peptides of human  $\beta$ -amyloid peptide<sup>1-40</sup>-OH and  $\beta$ -amyloid peptide<sup>25-35</sup>-OH can be purchased from Bachem, Torrance, California. Alternatively, the peptide can be synthesized by solid-phase fluorenylmethoxycarbonyl ("Fmoc") chemistry using techniques described, for example, in J.M. Stewart and J.D. Young, Solid-Phase Peptide Synthesis (2nd edition), pages 74-103 and 147-168, Pierce Chemical Company, Rockford, Illinois (1984); C.M. Deber, Peptides,

35

Structure and Function, pages 221-224 and 249-252, Pierce Chemical Company, Rockford, Illinois (1985); D.H. Schlesinger, Macromolecular Sequencing and Synthesis, Selected Methods and Applications, pages 153-220, Alan R. Liss, Inc., New York (1988); and G. R. Marshall, Peptides, Chemistry and Biology, pages 198-201, ESCOM Science Publishers, Netherlands (1988), the disclosures of which are incorporated by reference herein. It has been shown that the Fmoc strategy offers considerable advantages over the traditional Boc method for preparation of hydrophobic peptides (P. Rovero et al., Int. J. Peptide Protein Res. 37:140 (1991)). The resin used was polystyrene crosslinked with divinylbenzene and functionalized with an acid-labile linker. Sidechains were blocked with standard acid-labile blocking groups such as BOC, TMOB, and PMC. Alpha-amino groups were blocked with Fmoc. All activations were by diisopropylcarbodiimide and hydroxybenzotriazole in dichloromethane, except Gln and Asn, which were introduced as active (pentafluorophenyl) esters without further activation in dimethylformamide ("DMF") solution. Two hour couplings were used at each stage of the synthesis. A fourfold molar excess of amino acid monomer over peptide resin was used at each step. Removal of the Fmoc group after each coupling was achieved with 30% piperidine in 1:1 DMF:toluene for 5 and 15 minutes consecutively. Extensive washings of the resin between chemical steps was with both DMF and DMF:dichloromethane 1:1. Following the final coupling and deblocking cycle, the peptide resin was washed extensively with DMF, DMF:dichloromethane 1:1, and methanol. The peptides were cleaved from the resin using anhydrous trifluoroacetic acid containing 5% thianisole, 3% ethanedithiol, and 2% anisole as scavengers.

The peptides were purified to homogeneity by reverse-phase HPLC using a C<sub>18</sub>-column eluted with a

gradient of acetonitrile in 0.01 M aqueous TFA according to standard methods, such as those described in J.E. Shively, Methods of Protein Microcharacterization, A Practical Handbook, pages 3-88, Humana Press, Clifton, New Jersey (1986), and W.S. Hancock, CRC Handbook of HPLC for the Separation of Amino Acids, Peptides, and Proteins (Vol. II), pages 3-22, 279-286 and 303-312, CRC Press, Inc., Boca Raton, Florida (1987), the disclosures of which are incorporated by reference herein.

10 All peptides were further characterized by amino acid analysis and/or peptide sequencing according to standard methods such as those described in Shively supra. Peptides were stored at -20°C as dry lyophilizates or as stock solutions of  $10^{-3}$  M concentration in the solvents in which they were purified, i.e., in the HPLC solvents in which they eluted from the  $C_{18}$  HPLC column, with 1% 2-mercaptoethanol added as antioxidant. The composition of these solvents varied from 25% to 35% acetonitrile in aqueous 0.01 M TFA, with no evidence of peptide oxidation, aggregation, or degradation apparent over 4 months. In contrast, storage of the peptides in common solvents used for peptide stock solutions such as water, dimethylsulfoxide, glacial acetic acid, or 25 dimethylformamide gave significant oxidation, aggregation, or degradation resulting in materials not suitable for use in the procedures described below.

Except as otherwise noted, all peptides were based on the human  $\beta$ -amyloid peptide sequence.

30 Accordingly,  $\beta$ -amyloid peptide<sup>1-42</sup> is H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA-OH [SEQ. ID NO:2]. The analogous peptide in rat and mouse ( $G^5$ ,  $F^{10}$ ,  $R^{14}$ - $\beta$ -amyloid peptide<sup>1-42</sup>) differs at three positions within that sequence. Dutch  $\beta$ -amyloid peptide ( $Q^{22}$ - $\beta$ -amyloid peptide<sup>1-42</sup>) differs at one position within this 35 sequence. The sequence of  $\beta$ -amyloid peptide<sup>25-35</sup>-NH<sub>2</sub> is H-GSNKGAIIGLM-NH<sub>2</sub>.

Procedure for radioiodination of  $\beta$ -amyloid peptide<sup>1-40</sup>.

Peptides containing tyrosine were radiolabelled by oxidative radioiodination using Na<sup>125</sup>I and chloramine-T and separated from free iodide by reverse-phase absorption by modifications described hereinbelow of the methods of W.M. Hunter and F.C. Greenwood, Nature 194:495 (1962), A.E. Bolton and W.M. Hunter, Biochem. J. 133:529 (1973), and H.-P. Too and J.E. Maggio, Meth. Neurosci. 6:232 (1991), the disclosures of which are incorporated by reference herein. Peptides not containing tyrosine were first acylated with the N-hydroxysuccinimide ester of 4-hydroxyphenylpropionic acid, and then oxidatively radioiodinated as indicated hereinbelow. Briefly, labelled peptides containing methionine were then reduced from sulfoxide to native form with 2-mercaptoethanol. The iodinated peptides were purified by RP-HPLC to essentially quantitative specific activity (approximately 2000 Ci/mmol) and stored as described hereinabove at a concentration of less than 200,000 dpm/ $\mu$ l.

Acylation with the N-hydroxysuccinimide ester of 4-hydroxyphenylpropionic acid followed the method of H.-P. Too and J.E. Maggio, Meth. Neurosci. 6: 232 (1991), the disclosure of which is incorporated by reference herein. Care was taken to purify for future labelling only the monoacyl derivatives of the peptides, which were recognized by their elution profiles.

Standard radioiodination procedures such as those described in W.M. Hunter and F.C. Greenwood, supra, A.E. Bolton and W.M. Hunter, supra, and H.-P. Too and J.E. Maggio, supra, do not yield a viable tracer. It was found that iodination of the peptide must be performed at a high buffer salt concentration (M sodium phosphate, pH 7.5). However, since under those conditions solutions of the peptide are substantially unstable, it is necessary to perform the iodination step

quickly. Preferably the labelling reaction from the point of dissolving the peptide in the phosphate buffer to loading the reaction mixture onto an octyldecylsilica cartridge is completed within about one minute. If this  
5 part of the procedure is not completed in a sufficiently short period of time, the  $\beta$ -amyloid peptide will aggregate and fail to yield useful tracer.

It was found that successful iodination of the  $\beta$ -amyloid peptide requires starting with the peptide in  
10 its dry form. It was found that peptide placed in typical solvents such as aqueous buffer or dimethylsulfoxide did not yield a viable tracer. It was further found that the peptide remains stable in the solvent in which it is purified (35% acetonitrile in  
15 0.01 M aqueous TFA). Thus, the peptide (10 nmol) is loaded into the reaction vessel (a polypropylene microcentrifuge tube) by placing an aliquot of peptide solution in this solvent in the reaction vessel and then stripping the solvent in a vacuum centrifuge. It is  
20 preferred that a high molar ratio of peptide to radioiodine is used to minimize diiodination since the moniodinated form is preferred for use in the assays described herein.

It was found that the labelled peptide is  
25 viable as a tracer only when in the reduced (native methionine sidechain) form at high specific activity. Therefore additional steps of reduction and purification to high specific activity are necessary after the labelling reaction and its workup. Typical  
30 radioiodination syntheses are halted after labelling and the products used without additional steps. Such products, consisting of low specific activity peptides containing oxidized methionine, are acceptable for the majority of applications of peptide tracers such as  
35 radioimmunoassay. It was found that production of viable  $\beta$ -amyloid peptide tracer requires the additional

steps of reduction and purification as described hereinbelow.

It was determined that in order to avoid aggregation of the labeled peptide and consequent loss of viable tracer during the reduction reaction, the reaction time must be 90 minutes or less. Although reduction of the sulfoxide form is not complete at 90 minutes, the labelled  $\beta$ -amyloid peptide remains mostly as intact monomer. Longer reaction times provide more complete reduction at the risk of formation of unusable aggregates.

The purification of the moniodinated reduced tracer is accomplished by reverse-phase HPLC using a shallow gradient of acetonitrile in aqueous 0.01 M TFA which is capable of resolving oxidized from reduced forms, and uniodinated from moniodinated from diiodinated forms of the  $\beta$ -amyloid peptide. It is preferred that the reduced moniodinated form of the peptide is used in the assays described below.

To 10 nmol of dry  $\beta$ -amyloid peptide<sup>1-40</sup> in a polypropylene microcentrifuge tube is added 40  $\mu$ l of 0.5 M sodium phosphate pH 7.5 and 10  $\mu$ l (= 1 mCl) of aqueous Na<sup>125</sup>I, and the tube is vortexed briefly. Chloramine-T (10  $\mu$ l of 1 mg/ml in distilled water, freshly dissolved) is added to the mixture and the tube vigorously vortexed for 15 to 30 seconds. The reaction is then terminated by the addition of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (20  $\mu$ l of 10 mg/ml in distilled water), followed by brief vortexing.

The reaction mixture is immediately loaded onto an octyldecylsilica cartridge (volume approximately 0.5 ml) previously primed by washing with 3 ml acetonitrile containing 0.01 M TFA followed by 3 ml 0.01 M aqueous TFA. Examples of suitable octyldecylsilica cartridges include C<sub>18</sub> SPICE (Analtech), C<sub>18</sub> spe (Baker), and C<sub>18</sub> Sep-Pak (Waters). The octyldecylsilica cartridge is then eluted in step gradient fashion successively with 0.5 ml each of 0.01 M aqueous TFA containing 10%, 20%, and 40%

alcohol, and then eluted with 1 ml each of 80% and 100% alcohol, where alcohol is methanol:ethanol in a 1:1 volume. The labelled peptide of interest elutes in the 80% alcohol fraction. During elution of the cartridge, it is preferred that a slow flow rate is used, that air bubbles are avoided, and that the cartridge not be allowed to dry out to avoid adverse affects on the yield of labelled peptide.

The labelled peptide fraction which elutes from the octyldecylsilica cartridge includes the oxides of unlabelled, monoiodinated, and diiodinated peptides in alcoholic aqueous 0.01 M TFA. Chemical reduction to the native methionine forms is accomplished by concentrating the solution to less than about 25% of its original volume by gently evaporating the alcohol in a nitrogen stream, then adding neat 2-mercaptoethanol to a final concentration of 20%, and heating the resulting solution in a tightly capped tube under nitrogen at 90°C for 90 minutes. After cooling to room temperature, the mixture is purified by reverse-phase HPLC as described above, and the appropriate radioactive peptide (the reduced, monoiodinated form) retained for future use. Immediately following purification, 1% 2-mercaptoethanol is added to the purified tracer to prevent oxidation to the useless sulfoxide form. The tracer, now preferably at 2000 Ci/mmol (for  $^{125}\text{I}$ ), is stored as indicated above. At -20°C, solutions of less than 200,000 dpm/ $\mu\text{l}$  are stable for at least 4 months.

30

## EXAMPLE 2

### DETECTION OF *IN VITRO* $\beta$ -AMYLOID PEPTIDE DEPOSITION IN HUMAN TISSUE AND USE OF LABELLED $\beta$ -AMYLOID PEPTIDE

#### Preparation of tissue homogenates and thin sections.

Brain tissue was obtained from normal and Alzheimer's disease patients at 0.5 to 14 hours postmortem, frozen on dry ice after collection, and stored at -20°C until use. For filter binding studies, tissue homogenates

were prepared after the method of H.P. Too and M.R. Hanley, Biochem. J. 252: 545 (1988), the disclosure of which is incorporated by reference herein. Tissue was homogenized (Polytron, setting 7-8, 5-10 sec) in 5-10  
5 volumes of 50 mM Tricine (pH 7.5) containing 10% sucrose and protease inhibitors (0.01% bacitracin, 0.002% soybean trypsin inhibitor, 0.0002% chicken egg trypsin inhibitor, 1 mM benzamidine hydrochloride) and pelleted at 10,000 g for 20 minutes. The homogenate was then  
10 resuspended and washed several times in Tricine buffer containing 120 mM NaCl, 10 mM EDTA, 300 mM KCl and centrifuged at 40,000g for 20 minutes and stored at -20°C for less than two months. Membranes (equivalent to about 30 mg tissue) were resuspended in 0.5 ml 50 mM  
15 TrisHCl (pH 7.5) containing 1 mg/ml bovine serum albumin (BSA), 10 mM MnCl<sub>2</sub>, 0.004% bacitracin, 0.002% chymostatin, 0.004% leupeptin, 0.1% dimethylsulfoxide for 30 minutes prior to addition of the radioligand ( $10^{-11}$  to  $10^{-9}$  M) and various concentrations of unlabelled  
20 peptides in the same buffer.

After incubating for two hours at room temperature, the homogenates (final volume 0.575 ml) were filtered through glass fiber filters (Whatman GF/D), presoaked for at least two hours in 1 mg/ml BSA  
25 and rinsed with 25 mM Tricine (pH 7.5). After washing twice with 25 mM TrisHCl (pH 7.5) at room temperature, the filters were counted in a gamma counter. Signal/noise and specific binding were a function of the density of plaques in the tissue.

30 In Alzheimer's disease tissue homogenates, about 20,000 cpm (20 %) of the <sup>125</sup>I-labeled  $\beta$ -amyloid peptide<sup>1-40</sup> was bound in the absence of unlabelled peptides, and 10000 cpm (10%) in the presence of  $10^{-5}$  M unlabelled  $\beta$ -amyloid peptide<sup>1-40</sup>. No displacement binding  
35 was observed when plaques were absent (i.e., in normal tissue). Autoradiography of the homogenate confirmed



that the highest density of binding sites in Alzheimer's disease tissue was on intact plaques.

Autoradiography. For tissue autoradiography, unfixed tissue was serially sectioned at 5-15  $\mu\text{m}$  and thaw-mounted onto gelatin-coated slides. Slide-mounted sections were stored at  $-20^{\circ}\text{C}$  in closed boxes over desiccant for less than three months before use. Sections were preincubated for 30 minutes and incubated with the radioligand for two hours under the same conditions according to the aforescribed homogenate binding study. Alternatively, 50mM TrisHCl rather than Tricine may be used as the buffer. For estimating nonspecific binding, paired serial sections were incubated with the radioligand in the presence of a  $10^4$  to  $10^5$  fold excess of the unlabelled peptide. Following incubation with the radioligand, the slides were washed with 50 mM TrisHCl pH 7.5 (four times, two minutes each at  $4^{\circ}\text{C}$ ), then dried at  $4^{\circ}\text{C}$  and stored in closed boxes over desiccant at room temperature overnight. The fully dried slides were then placed in apposition to tritium-sensitive film alongside iodinated standards. After one week's exposure at  $-20^{\circ}\text{C}$ , the film was developed, fixed and washed. Sections were later dipped in photographic emulsion for higher resolution autoradiography and/or counterstained by standard procedures with Congo red, thioflavin S, creosyl violet, hematoxylin and eosin, or antibodies for immunohistochemical analysis. This approach generated film autoradiograms for quantitative densitometry, a high resolution emulsion autoradiograms for detailed histology, and a counterstained section for identification of cell types from each tissue section.

RESULTS. While there was essentially no displaceable binding of the radioligand to normal tissue homogenates or sections, there was significant displaceable binding to Alzheimer's disease tissue (see Figures 1 and 2).

The binding to Alzheimer's disease tissue was not saturable, suggesting that most of the sites to which the radioligand bound were not receptors in the usual sense (i.e., receptor directly coupled to an effector mechanism that directly affects the intra-cellular environment). Rather, the characteristics of this binding were consistent with growth of Alzheimer's disease amyloid plaques by deposition of  $\beta$ -amyloid peptide from solution.

10 Radiolabelled human  $\beta$ -amyloid peptide was deposited in vitro from dilute ( $<10^{-11}$  M) solution onto neuritic, diffuse, and cerebrovascular plaques in AD brain tissue, within 30 to 60 minutes. In tissue without preformed plaques, no deposition was detected. 15 These results indicate that all three types of plaques are capable of growth through deposition of exogenous amyloid peptide in the presence of very low amounts of  $\beta$ -amyloid peptide. These results further indicate that plaque growth alone does not explain the selective 20 damage to particular subsets of neurons which typifies the disease process.

Visualization of the binding sites for  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> by autoradiography showed that the ligand was deposited on amyloid plaques at both 25 parenchymal and vascular sites in Alzheimer's disease brain. Thus, in the Alzheimer's disease cerebral cortex,  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> was deposited on both the core and the halo of essentially every extracellular plaque examined (Figure 2). In the Alzheimer's disease 30 cerebellar cortex, diffuse plaques which were not visualized with thioflavin S were readily labelled with the  $\beta$ -amyloid peptide radioligand (see Figure 2), and clear morphological differences between these plaques and the compact plaques of the cerebral cortex were 35 evident. Thus, both the classic senile plaques of the cortex and the diffuse nonneuritic deposits of the cerebellum were found capable of in vitro growth by

addition of  $\beta$ -amyloid peptide from dilute solution. While thioflavin S and anti-A4 antibodies stained neurons outside the plaques as well as the plaques themselves, deposition of the radioligand was limited to the plaques alone (see Figure 2). Furthermore, the sensitivity of detection of plaques with the radioligand far exceeded that of dyes or antibodies. Thus, the radioligand was capable of detecting more lesions at a potentially earlier time than detection techniques reported to date. In cerebral cortex tissue obtained from normal brain, there was essentially no deposition of  $\beta$ -amyloid peptide radioligand detected above background levels at parenchymal or vascular sites.

Vascular plaques were visualized by  $\beta$ -amyloid peptide deposition in approximately 0-20% of intra- and extraparenchymal blood vessels in Alzheimer's disease brain, although the fraction of vessels labelled showed considerable variation between cortical areas examined and between patients (See Table II). The cerebrovascular deposits were consistently labelled more densely than cerebral plaques within the same section. In vessels seen in transverse section (see Figure 3), the deposition of  $\beta$ -amyloid peptide radioligand was not uniform but concentrated in a part of the vessel, apparently the tunica media. Endothelial tissue was not labeled.

Deposition of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> onto plaques in both homogenates and sections of Alzheimer's disease tissue was significantly attenuated by excess unlabelled  $\beta$ -amyloid peptide<sup>1-40</sup> and Dutch  $\beta$ -amyloid peptide<sup>1-40</sup>, as addition of these unlabelled peptides to the plaques competed with deposition of the radioligand (see Figure 1).  $\beta$ -amyloid peptide<sup>25-35</sup>-NH<sub>2</sub> also competed with the 40-mer radioligand when the former was present at higher concentrations (see Figure 1), while the free acid  $\beta$ -amyloid peptide<sup>25-35</sup>-OH had no detectable activity in the assay. Thus, the growth of amyloid plaques

in vitro required only the presence of  $\beta$ -amyloid peptide in the surrounding media. The affinity of the amyloid peptide for the plaques was sufficiently high that even when the concentration of  $\beta$ -amyloid peptide<sup>1-40</sup> was below  $10^{-11}$  M, deposition occurred. No significant differences in  $\beta$ -amyloid peptide deposition were noted between homogenates and sections of Alzheimer's tissue nor were any significant differences noted between Alzheimer's disease cerebral cortex (neuritic plaques) and cerebellum (diffuse plaques). These results were consistent with the hypothesis that the plaques themselves (neuritic, diffuse, and vascular) can grow in vivo in the presence of  $\beta$ -amyloid peptide.

The mammalian tachykinins, substance P and neurokinins A and B, over a broad range of concentration, did not inhibit the deposition of radiolabelled  $\beta$ -amyloid peptide onto Alzheimer's disease plaques (see Figure 1). Binding sites for radiolabelled tachykinins were present in both Alzheimer's disease and normal tissue, and were not associated with plaques. These tachykinin binding sites were indistinguishable from the tachykinin receptors that have been described by J.E. Maggio, Ann. Rev. Neurosci., 11:13 (1988), and P.W. Mantyh et al., Proc. Natl. Acad. Sci., 86:5193 (1989), with unlabelled tachykinins displacing their radiolabelled analogues at nanomolar concentrations. In contrast, there was no displacement of any of the tachykinin radioligands by  $\beta$ -amyloid peptide<sup>1-40</sup> at concentrations up to 30  $\mu$ M, which indicates that the amyloid peptide does not interact with tachykinin receptors under the standard conditions labelling tachykinin receptors as described. These results were consistent with the reported structure-activity studies among the tachykinin peptide family, namely, that a carboxyl-terminal amide is required for activity.

Radiolabelled rat  $\beta$ -amyloid peptide<sup>1-40</sup> failed to bind to Alzheimer's disease or normal human tissue, or

to adult rat brain. In addition, unlabelled rat  $\beta$ -amyloid peptide<sup>1-40</sup> did not inhibit deposition of <sup>125</sup>I- $\beta$ -amyloid peptide<sup>1-40</sup> onto Alzheimer's disease plaques. These results are consistent with the observation that rodents do not develop amyloid plaques, and indicates that the sequence of the amyloid peptide itself is important in plaque genesis and growth.

The avidity of  $\beta$ -amyloid peptide for amyloid plaques indicates that once an aggregate of amyloid peptide has formed, even extremely low concentrations of  $\beta$ -amyloid peptide will support its growth. It was found that neuritic, diffuse, and vascular plaques were indistinguishable in this capacity. Since neuritic plaques in the cerebral cortex were often surrounded by dying neurons while diffuse plaques in the cerebellum were not, these results indicate that if  $\beta$ -amyloid peptide is neurotoxic, it is selectively neurotoxic to a subset of central neurons.

The use of radioiodinated  $\beta$ -amyloid peptide provides an in vitro system for the quantitative evaluation of agents or conditions which may inhibit or enhance the growth of plaques, a sensitive method for visualizing various types of amyloid deposits, a means for characterizing and locating sites of amyloid peptide binding to cells and tissues, and for investigation of the role of amyloid deposits in the pathogenesis of Alzheimer's disease.

### EXAMPLE 3

#### IN VITRO EVALUATION OF AGENTS FOR INHIBITING OR ENHANCING

#### AGGREGATION OF $\beta$ -AMYLOID PEPTIDE, OR FOR DISPERSING AGGREGATES OF $\beta$ -AMYLOID PEPTIDE, IN THE ABSENCE OF ALZHEIMER'S DISEASE PLAQUES

The experiments described in Example 1 demonstrated that amyloid plaques can grow in vitro by deposition of labeled amyloid peptide from dilute solution. The following experiment with radiolabelled

$\beta$ -amyloid peptide demonstrated that the peptide can aggregate in vitro in the absence of amyloid plaques. This latter property provides an in vitro system for qualitative and quantitative evaluation of agents or conditions that may inhibit or enhance this aggregation or disperse preformed aggregates. Agents so identified may have similar effects on Alzheimer's disease plaques in vivo.

A solution of about  $10^{-9}$  M labelled  $\beta$ -amyloid peptide in 50 mM Tricine buffer at pH 7.5 was prepared from stock solution of the peptide as described hereinabove in Example 1. The solution was aliquotted into several reaction vessels (polypropylene microcentrifuge tubes) and allowed to stand at room temperature with occasional vortex mixing. At various times, the tubes were centrifuged at 12000g for 4 minutes, and the fraction of initial ( $t=0$ ) cpm of labelled peptide remaining in the supernatant fraction determined by removing a small aliquot for counting. The time course of the disappearance of the tracer from the supernatant under these conditions is shown in Figure 4. The rate of disappearance was dependent on a variety of other conditions which were evaluated using this assay. Thus, the rate of the disappearance depends on peptide concentration (faster at higher peptide concentrations), and on ionic strength (faster at higher salt concentrations). The rate of disappearance was further dependent on the presence of certain detergents such as sodium dodecylsulfate (SDS) (faster in 0.01% SDS; see, Figure 4), and on the presence of certain organic solvents (slower in the presence of acetonitrile).

Methods similar to those described hereinabove may be used to assay the rate of formation of aggregates which may be separated by filtration or centrifugation, or the rate of dispersion of aggregates of amyloid peptide, or the effects of various agents on these

processes. In each case, a key step is the use of labelled amyloid peptide in the aggregate or in solution to follow the time course of the process.

5

#### EXAMPLE 4

##### IN VITRO EVALUATION OF AGENTS FOR INHIBITING OR ENHANCING PLAQUE GROWTH

The competitive binding assay, as described in Figure 1, was conducted to determine inhibition of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> aggregation by amyloid and tachykinin peptides in homogenates of Alzheimer's disease temporal cortex. The aggregation affecting agents that were tested included  $\beta$ -amyloid peptide<sup>1-40</sup>,  $\beta$ -amyloid peptide<sup>25-35</sup>-NH<sub>2</sub>,  $\beta$ -amyloid peptide<sup>25-35</sup>-OH, rat  $\beta$ -amyloid peptide<sup>1-40</sup>, substance P, and neurokinins A and B.

As shown in Table II, patients considered to have Alzheimer's disease were clinically diagnosed as such, and contained numerous plaques, as determined by thioflavin S staining of brain tissue samples. Control subjects were age-matched patients with no history of dementia.

Tissue homogenates of temporal cortex tissue obtained from the control subjects were prepared according to the protocol set forth in Example 1. Autoradiography, also as set forth in Example 1, indicated the absence of amyloid plaques in the homogenized tissue material. (See, Table II). As the tissue samples displayed no evidence of Alzheimer's disease, the samples were considered "normal" tissue, and used as controls.

Tissue homogenates of temporal cortex tissue obtained from patients with Alzheimer's disease were also prepared according to Example 1 (See, Table II). Autoradiography detected amyloid plaques in the homogenized tissue material.

Inhibition of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> deposition was determined by adding increasing

concentrations of the deposition affecting agents/peptides to the incubation medium and determining the percent inhibition of deposition. Figure 1 shows that whereas  $\beta$ -amyloid peptide<sup>1-40</sup> or Dutch  $\beta$ -amyloid peptide<sup>1-40</sup> are potent inhibitors of <sup>125</sup>I- $\beta$ -amyloid peptide<sup>1-40</sup> deposition,  $\beta$ -amyloid peptide<sup>25-35</sup>-NH<sub>2</sub> is substantially less potent and substance P, Neurokinin A and B,  $\beta$ -amyloid peptide<sup>25-35</sup>-OH, and rat  $\beta$ -amyloid peptide<sup>1-40</sup> are essentially inactive. This shows the usefulness of this assay in assessing an agent's ability to inhibit  $\beta$ -amyloid peptide<sup>1-40</sup> deposition to pre-formed plaques.

#### EXAMPLE 5

##### 15 LOCALIZATION OF <sup>125</sup>I- $\beta$ -AMYLOID PEPTIDE<sup>1-40</sup> BINDING SITES IN ALZHEIMER'S DISEASE BRAIN TISSUE

Tissue sections of Alzheimer's disease temporal cortex from Example 3 were examined by autoradiography to detect binding sites of <sup>125</sup>I- $\beta$ -amyloid peptide<sup>1-40</sup>.

There was no specific deposition of  $\beta$ -amyloid peptide<sup>1-40</sup> in the absence of plaques. As shown in Figure 2, an autoradiograph of <sup>125</sup>I- $\beta$ -amyloid peptide<sup>1-40</sup> binding in tissue sections of Alzheimer's disease temporal cortex, tissue from areas without plaques showed no binding of <sup>125</sup>I- $\beta$ -amyloid peptide<sup>1-40</sup> which could be displaced by excess  $\beta$ -amyloid peptide<sup>1-40</sup>.

A dark field photomicrograph showing the distribution of <sup>125</sup>I- $\beta$ -amyloid peptide<sup>1-40</sup> in Alzheimer's disease temporal cortex revealed numerous plaques throughout the grey matter (Figure 2(a)). A dark-field micrograph of a serially adjacent section as treated in Figure 2(a), except that 10<sup>-5</sup>M cold  $\beta$ <sub>1-40</sub> was added to the incubation medium, is shown in Figure 2(b). An immunohistochemistry of amyloid deposits using antibodies raised against  $\beta$ -amyloid peptide<sup>1-40</sup> (amyloid peptide A4) in Alzheimer's disease temporal cortex is shown in Figure 2(c). Figure 2(d) is a dark-field



photomicrograph of the same section as shown in Figure 2(c), where  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> revealed a more extensive distribution of plaques than did the anti-A4 antibody. Figure 2(e) is a dark-field photomicrograph of thioflavin S staining in human Alzheimer's disease temporal cortex showing labelling of diffuse, compact and neuritic type plaques. Figure 2(f) is a light-field photomicrograph of the same section as Figure 2(e) bound with  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup>, showing that all three types of plaques bind  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup>. Figure 2(g) is a dark-field photomicrograph of thioflavin S staining in human Alzheimer's disease temporal cortex showing labelling of a neuritic plaque and several adjacent neurons. Figure 2(f) is a light-field photomicrograph showing the same section as Figure 2(g), bound with  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup>, showing that although both the core and halo of the plaque bind  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup>, none of the labeled neurons show any  $^{125}\text{I}$ - $\beta$ -amyloid peptide binding.

Localization of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> binding sites in the cerebral vasculature of Alzheimer's disease brain is shown in Figure 3. Figure 3(a) shows the distribution of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> in plaques in the parenchyma and in blood vessels (BV) in Alzheimer's disease temporal cortex. Figure 3(b) shows a serially adjacent section treated in the as that of Figure 3(a), except that  $5.0 \times 10^{-5}\text{M}$  cold  $\beta$ -amyloid peptide<sup>1-40</sup> was added in the incubation medium. Figure 3(c) is a light-field photomicrograph showing the localization of  $^{125}\text{I}$ - $\beta$ -amyloid peptide<sup>1-40</sup> in a cerebral artery. Figure 3(d) is a dark-field photomicrograph of the same section as in Figure 3(c) showing the binding of  $^{125}\text{I}$ - $\beta$ -amyloid peptide over the tunica media of the cerebral artery.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many

variations and modifications may be made while remaining within the spirit and scope of the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Mantyh, Patrick W.  
Maggio, John E.
- (ii) TITLE OF INVENTION: Labelled  $\beta$ -Amyloid Peptide  
and Alzheimer's Disease Detection
- (iii) NUMBER OF SEQUENCES: 2
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Merchant & Gould
  - (B) STREET: 3100 Norwest Center
  - (C) CITY: Minneapolis
  - (D) STATE: Minnesota
  - (E) COUNTRY: USA
  - (F) ZIP: 55402
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette, 3.5 inch, 720 Kb
  - (B) COMPUTER: Northgate 386
  - (C) OPERATING SYSTEM: DOS 4.0
  - (D) SOFTWARE: WordPerfect<sup>®</sup> 5.0
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 13-Aug-1991
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Kowalchuk, Alan W.
  - (B) REGISTRATION NUMBER: 31,535
  - (C) REFERENCE/DOCKET NUMBER: 600.226-WO-01
- (xi) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (612) 332-5300
  - (B) TELEFAX: (612) 332-9081

(2) INFORMATION FOR SEQUENCE ID NO: 1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 40 amino acid residues
  - (B) TYPE: Amino Acid
  - (D) TOPOLOGY: Linear

- (ii) MOLECULE TYPE: Peptide
- (v) FRAGMENT TYPE: Internal Fragment
- (vi) ORIGINAL SOURCE: Synthetically Derived
- (ix) FEATURE:
  - (A) NAME/KEY: Internal fragment of the  $\beta$ -amyloid peptide precursor
  - (B) LOCATION: Represents isolated internal sequence of 40 amino acid residues from the  $\beta$ -amyloid peptide precursor
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
 

Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val  
   1                  5                  10

His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val  
           15                  20

Gly Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val  
  25                  30                  35

Gly Gly Val Val  
                   40

(2) INFORMATION FOR SEQUENCE ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 42 amino acid residues
  - (B) TYPE: Amino Acid
  - (D) TOPOLOGY: Linear
- (ii) MOLECULE TYPE: Peptide
- (v) FRAGMENT TYPE: Internal Fragment
- (vi) ORIGINAL SOURCE: Synthetically Derived
- (ix) FEATURE:
  - (A) NAME/KEY: Internal fragment of the  $\beta$ -amyloid peptide precursor
  - (B) LOCATION: Represents isolated internal sequence of 40 amino acid residues from the  $\beta$ -amyloid peptide precursor

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Asp	Ala	Glu	Phe	Arg	His	Asp	Ser	Gly	Tyr	Glu	Val
1				5					10		
His	His	Gln	Lys	Leu	Val	Phe	Phe	Ala	Glu	Asp	Val
		15				20					
Gly	Ser	Asn	Lys	Gly	Ala	Ile	Ile	Gly	Leu	Met	Val
25				30					35		
Gly	Gly	Val	Val	Ile	Ala						
		40		42							

WHAT IS CLAIMED IS:

1. An in vitro method of detecting Alzheimer's disease in a patient, comprising:
  - (a) combining a sample of tissue from said patient, with an amount of labelled  $\beta$ -amyloid peptide or active fragment effective to bind with tissue evidencing the presence of, or susceptible to, Alzheimer's disease, for a time effective to allow binding of the labelled peptide or peptide fragment with the tissue; and
  - (b) detecting the presence of labelled peptide or peptide fragment bound to the tissue sample.
2. The method according to claim 1, further comprising:
  - (c) quantifying the amount of labelled peptide or peptide fragment bound to the tissue sample.
3. The method according to claim 2, further comprising monitoring the progression of Alzheimer's disease in the patient, comprising:
  - (d) combining a later-acquired sample of tissue from said patient with an amount of labelled  $\beta$ -amyloid peptide or active fragment thereof for a time effective to allow binding of the labelled peptide or fragment with the tissue;
  - (e) quantifying the amount of labelled peptide or peptide fragment bound to the later-acquired tissue sample;
  - (f) comparing the amount of bound peptide or peptide fragment in the tissue sample from step (b) with the amount of bound peptide or peptide fragment in the later-acquired tissue sample.

4. The method according to claim 1, wherein the  $\beta$ -amyloid peptide has the amino acid sequence  
H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-OH [SEQ. ID NO:1].
5. The method according to claim 1, wherein the label of the  $\beta$ -amyloid peptide or peptide fragment is radioactive, enzymatic, fluorescent or any combination thereof.
6. The method according to claim 1, wherein the bound tissue labeled peptide complexes are analyzed by autoradiography, positron emission tomography, nuclear magnetic resonance imaging, a gamma counter or a scintillation counter.
7. An in vivo method of detecting Alzheimer's disease in a patient, comprising:
  - (a) administering to said patient a labelled  $\beta$ -amyloid peptide or active fragment thereof in an amount effective to bind with tissue evidencing the presence of, or susceptible to, Alzheimer's disease; and
  - (b) detecting the presence of the labelled peptide or peptide fragment bound to tissue in the patient.
8. The method according to claim 7, wherein bound tissue labeled peptide complexes are detected by positron emission tomography.
9. The method according to claim 7, further comprising measuring the progression of Alzheimer's disease in the patient, comprising:

- (d) administering labelled  $\beta$ -amyloid peptide or active fragment thereof to a patient in an amount effective to bind with tissue evidencing the presence of, or susceptible to, Alzheimer's disease, wherein the labelled peptide or peptide fragment is administered at a time later than step (a);
  - (e) quantifying the amount of the later-administered, tissue-bound labelled peptide or peptide fragment; and
  - (f) comparing the amount of tissue-bound peptide or peptide fragment from step (b) with the amount of later-administered, tissue-bound peptide or peptide fragment from step (e).
10. An in vitro method of screening an agent capable of affecting the aggregation of  $\beta$ -amyloid peptides, comprising:
- (a) combining  $\beta$ -amyloid peptide or peptide fragment thereof with a potential aggregation affecting agent to be screened and an acceptable buffer or solvent to provide a solution;
  - (b) detecting the amount of  $\beta$ -amyloid peptide or peptide fragment aggregation on the solution; and
  - (c) assessing the effect of the agent on  $\beta$ -amyloid peptide or peptide fragment aggregation.
11. The method of claim 10 wherein said solution includes an aggregation enhancing agent.

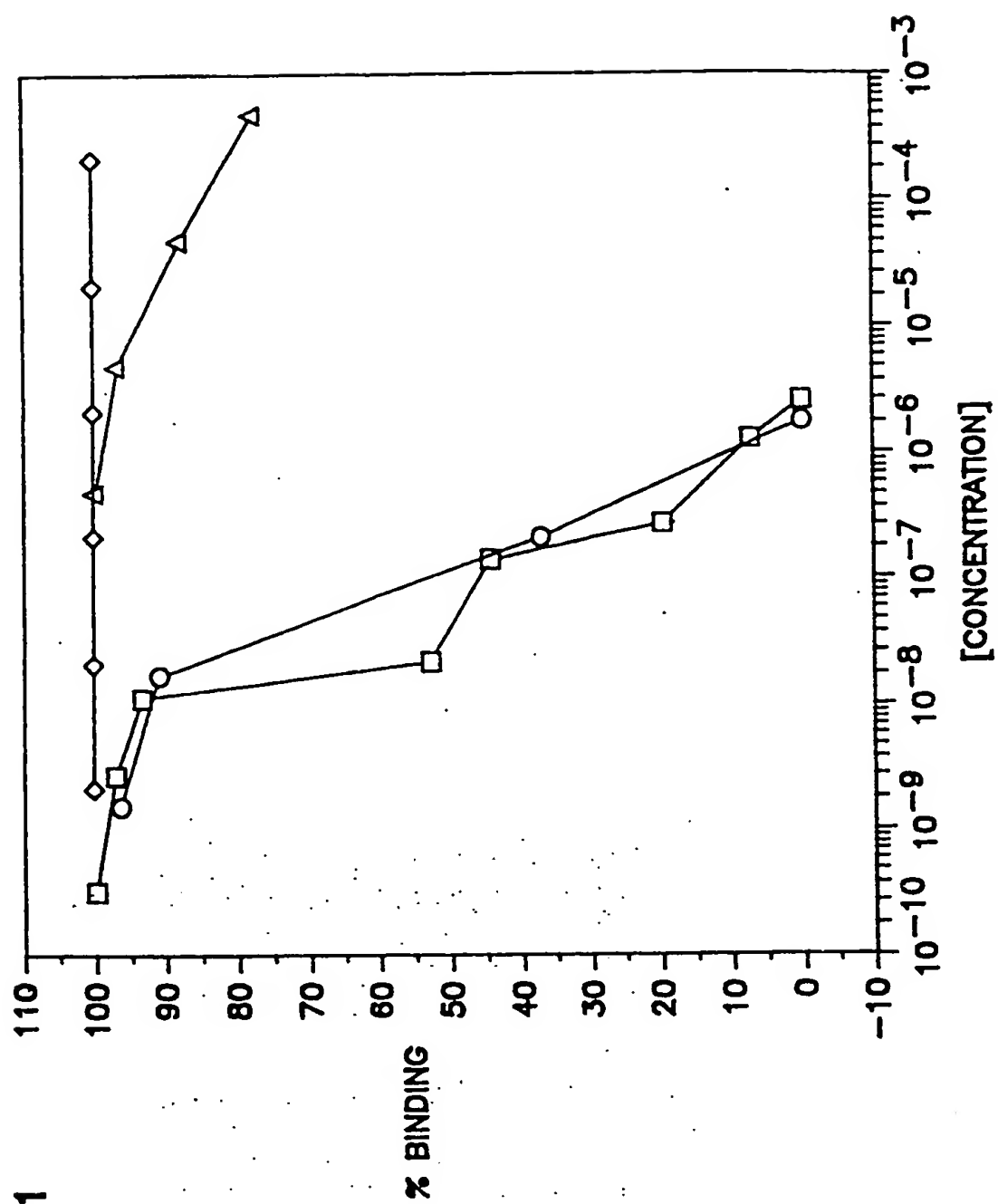


12. The method according to claim 10, wherein the agent is capable of inhibiting aggregation of  $\beta$ -amyloid peptide.
13. The method according to claim 10, wherein the agent is capable of enhancing aggregation of  $\beta$ -amyloid peptide.
14. An in vitro method of screening an agent capable of affecting the aggregation of  $\beta$ -amyloid peptide, comprising:
  - (a) combining  $\beta$ -amyloid peptide or peptide fragment thereof with an aggregation enhancing agent in an acceptable buffer or solvent to provide a solution, the aggregation enhancing agent being present in an amount effective to initiate aggregation of the peptide or peptide fragment;
  - (b) adding to the solution a potential aggregation affecting agent to be screened for effect on aggregation of the peptide or peptide fragment;
  - (c) detecting the amount of  $\beta$ -amyloid peptide or peptide fragment aggregation in the solution; and
  - (d) assessing the effectiveness of the potential aggregation affecting agent to affect the amount of  $\beta$ -amyloid peptide or peptide fragment aggregation.
15. The method according to claim 14, wherein the  $\beta$ -amyloid peptide has the amino acid sequence  
H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-OH [SEQ. ID NO:1].

16. The method according to claim 14, wherein the aggregation enhancing agent is a pre-formed aggregate of the peptide or peptide fragment, amyloid plaque derived from Alzheimer's disease tissue, or a detergent or metal ion capable of expediting the aggregation.
17. An in vitro method of screening an agent capable of affecting the deposition of  $\beta$ -amyloid peptide on tissue, comprising:
- (a) combining a sample of tissue from a patient with an amount of labelled  $\beta$ -amyloid peptide or active fragment effective to bind with tissue evidencing the presence of, or susceptible to, Alzheimer's disease, and with a potential deposition affecting agent to be screened, for a time effective to allow binding of the labelled peptide or peptide fragment with the tissue;
  - (b) detecting the amount of labelled peptide or peptide fragment bound to the tissue sample; and
  - (c) assessing the effect of the agent on  $\beta$ -amyloid peptide or peptide fragment deposition.
18. A composition comprising labelled  $\beta$ -amyloid peptide, said peptide having the amino acid sequence  
H-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-OH [SEQ. ID NO:1].
19. A pharmaceutical composition for use in detecting Alzheimer's disease in a human tissue, comprising:
- (a) labelled  $\beta$ -amyloid peptide or active peptide fragment thereof, in combination with a

pharmaceutically acceptable carrier, the peptide or peptide fragment having the ability to bind to tissue evidencing the presence of, or susceptible to, Alzheimer's disease.

20. The composition of claim 19, wherein said labelled peptide or peptide fragment is radiolabelled.
21. The composition of claim 20, wherein said peptide or peptide fragment is radiolabelled with radioactive iodine.



SUBSTITUTE SHEET

FIG. 2 A

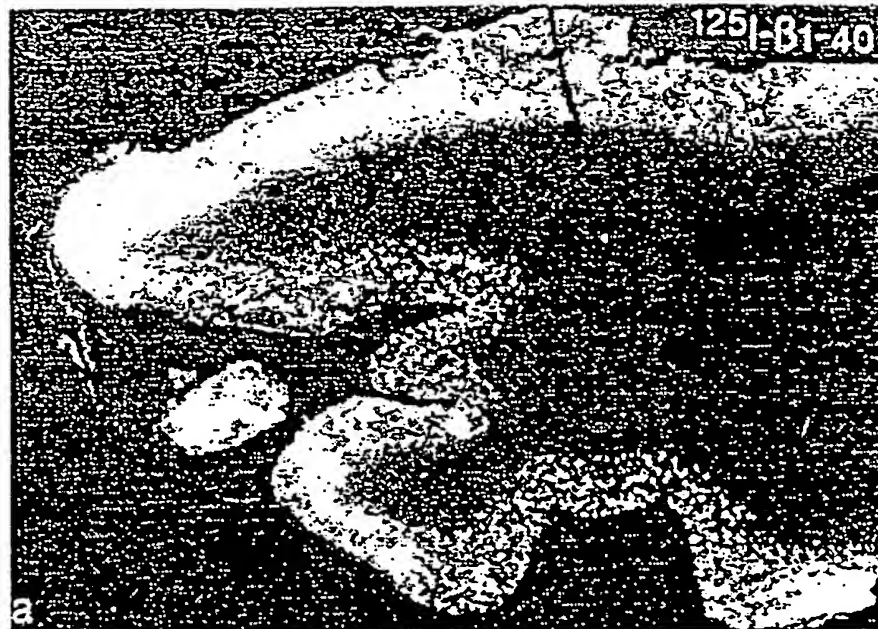
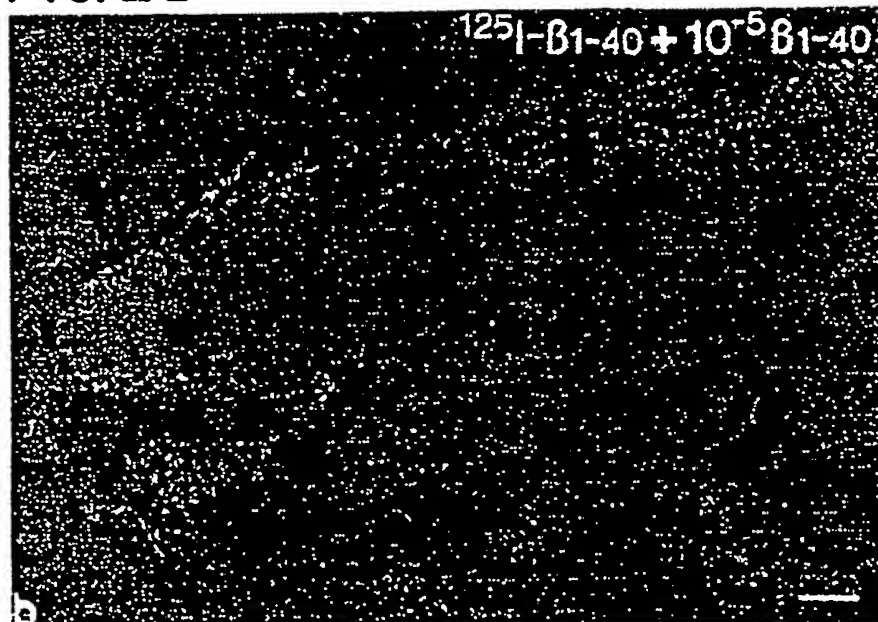


FIG. 2 B



SUBSTITUTE SHEET

FIG. 2C

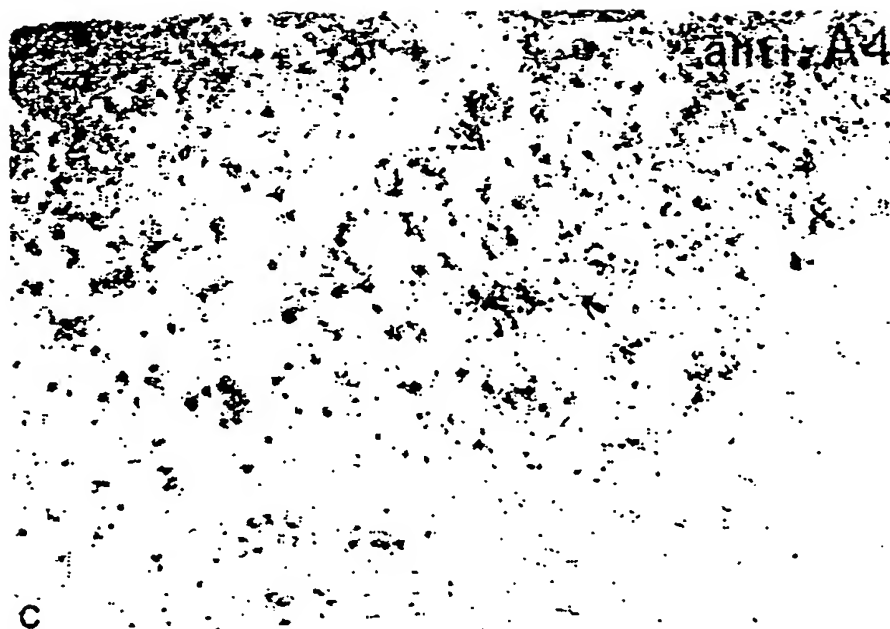


FIG. 2D



SUBSTITUTE SHEET

FIG. 2E

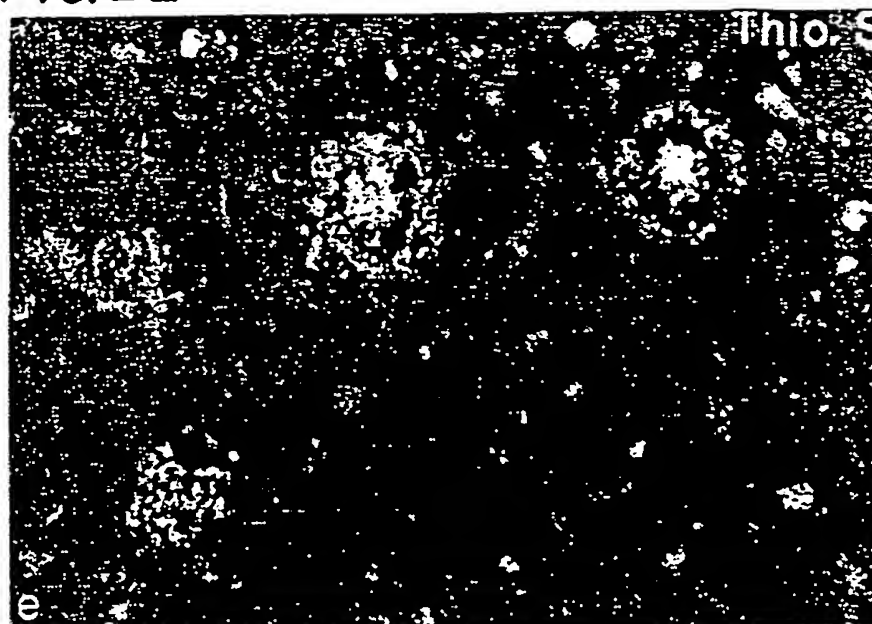
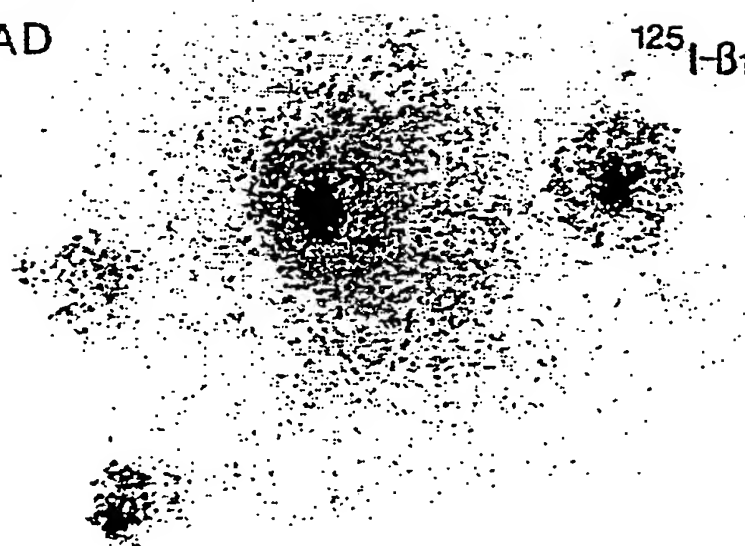


FIG. 2F

AD

125 I-B1-40



SUBSTITUTE SHEET

FIG. 2G

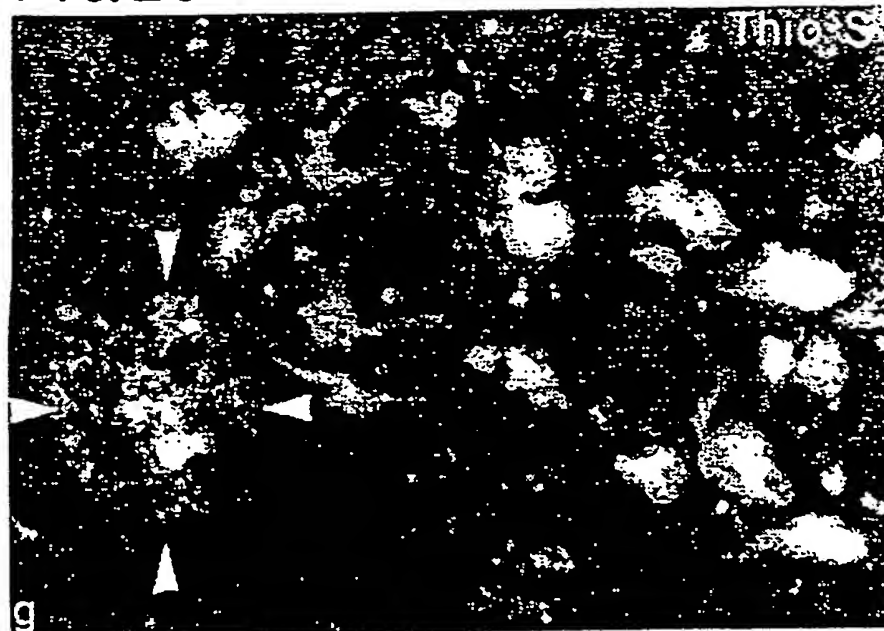


FIG. 2H



SUBSTITUTE SHEET



FIG. 3A

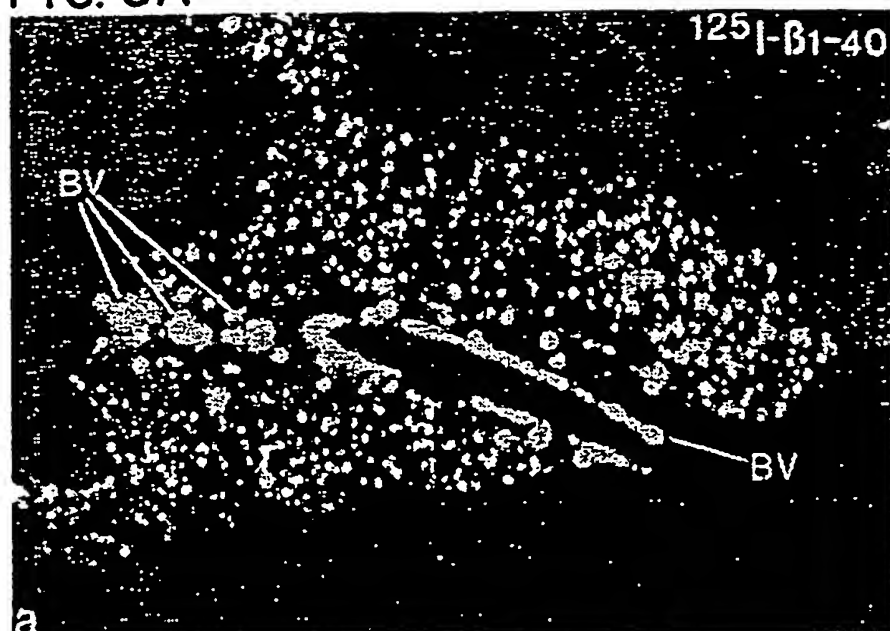
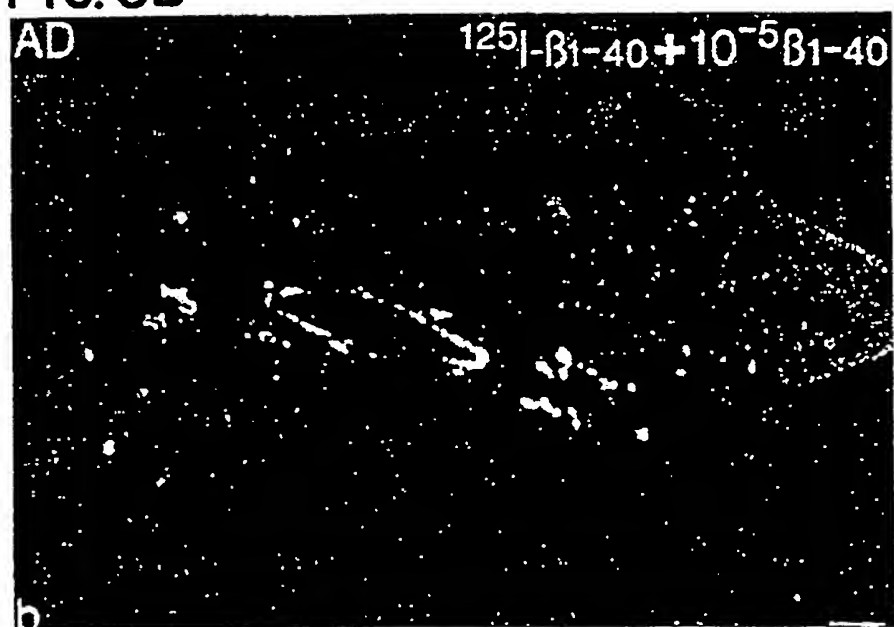


FIG. 3B

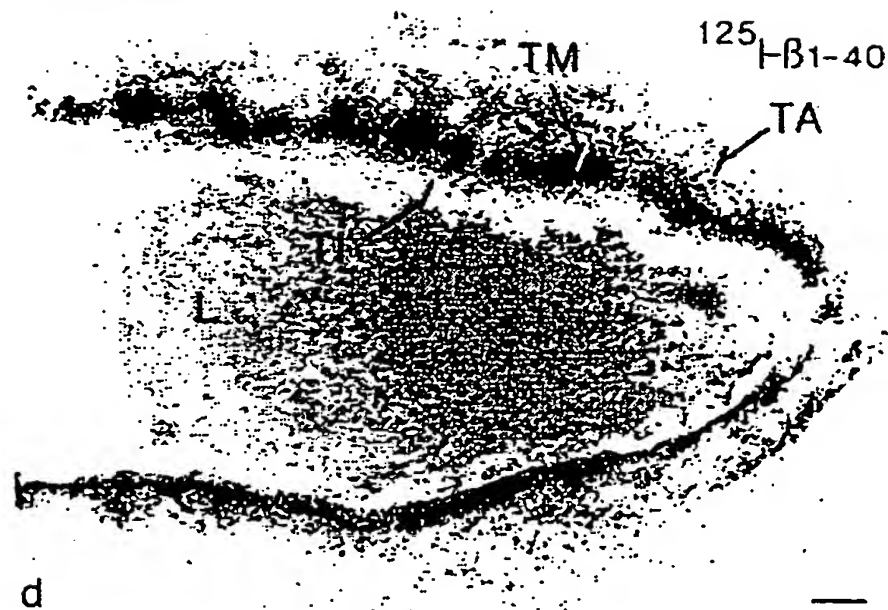


SUBSTITUTE SHEET

FIG. 3C



FIG. 3D



SUBSTITUTE SHEET

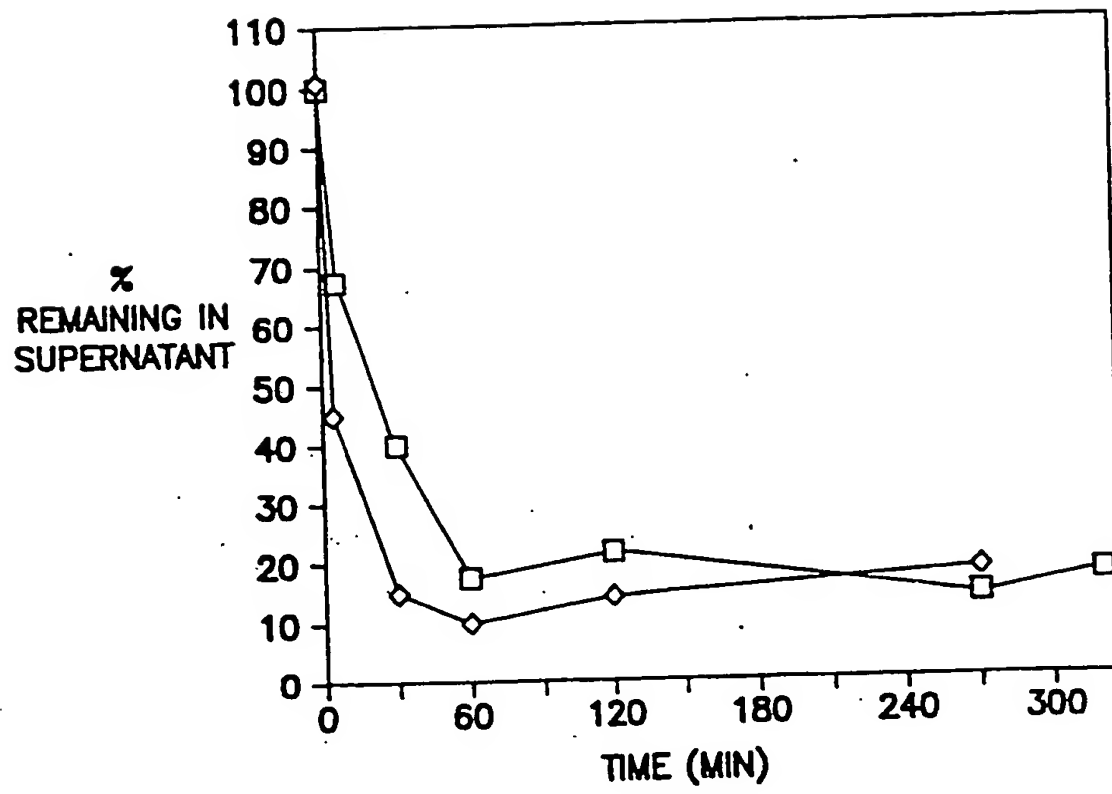


FIG. 4

SUBSTITUTE SHEET

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/06700

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12Q 1/25; G01N 33/53; A61K 35/30

US CL : 435/5, 7.2; 436/518, 548; 424/85.8; 530/387.1, 388.2, 324

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.2; 436/518, 548; 424/85.8; 530/387.1, 388.2, 324

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, APS, DIALOG, SEQUENCE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOLUME 85, ISSUED APRIL 1988, D. ALLSOP ET AL., "IMMUNOHISTOCHEMICAL EVIDENCE FOR THE DERIVATION OF A PEPTIDE LIGAND FROM THE AMYLOID $\beta$ -PROTEIN PRECURSOR OF ALZHEIMER DISEASE", PAGES 2790-2794, ENTIRE DOCUMENT.	<u>1, 5, 19</u> 2-4, 6, 18-21
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, VOLUME 84, ISSUED JUNE 1987, N. K. ROBAKIS ET AL., "MOLECULAR CLONING AND CHARACTERIZATION OF A cDNA ENCODING THE CEREBROVASCULAR AND THE NEURITIC PLAQUE AMYLOID PEPTIDES", PAGES 4190-4194, ESPECIALLY PAGE 4192.	1-21
Y	WO, A, 90/05138 (NEVE ET AL.) 17 MAY 1990, SEE ABSTRACT AND PAGE 12.	3
Y	US, A, 4,666,829 (GLENNER ET AL.) 19 MAY 1987, ENTIRE DOCUMENT	1-21
Y	THE EMBO JOURNAL, VOLUME 8, NUMBER 2, ISSUED 1989, M. GOEDERT ET AL., "CLONING AND SEQUENCING OF THE cDNA ENCODING AN ISOFORM OF MICROTUBULE-ASSOCIATED PROTEIN TAU CONTAINING FOUR TANDEM REPEATS: DIFFERENTIAL EXPRESSION OF TAU PROTEIN mRNAs IN HUMAN BRAIN", PAGES 393-399, ENTIRE DOCUMENT.	1-21

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	* T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* A* document defining the general state of the art which is not considered to be part of particular relevance	* X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
* E* earlier document published on or after the international filing date	* Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
* L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* &*	document member of the same patent family
* O* document referring to an oral disclosure, use, exhibition or other means		
* P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

17 SEPTEMBER 1992

Date of mailing of the international search report

30 SEP 1992

Name and mailing address of the ISA/  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

BETTY J. HAYDEN, PH.D.

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)\*